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(71) Applicant *(for all designated States except US)*: HUMAN GENOME SCIENCES, INC. [US/US]; Suite 300, 9620 Medical Center Drive, Rockville, MD 20850-3338 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LI, Haodong [CN/US]; 11033 Rutledge Drive, Gaithersburg, MD 20878 (US). ADAMS, Mark, D. [US/US]; 15205 Dufief Drive, North Potomac, MD 20878 (US).

(74) Agents: OLSTEIN, Elliot, M., et al.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US).

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(54) Title: CONNECTIVE TISSUE GROWTH FACTOR-2

(57) Abstract

The present invention relates to a human CTGF-2 polypeptide and DNA (RNA) encoding such polypeptide. Also provided is a procedure for producing such polypeptide by recombinant techniques and antibodies and antagonist/inhibitors against such polypeptide. Also provided are methods of using the polypeptide therapeutically for enhancing the repair of connective and support tissue, promoting the attachment, fixation and stabilization of tissue implants and enhancing wound healing.

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CONNECTIVE TISSUE GROWTH FACTOR-2

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. More particularly, the polypeptide of the present invention is connective tissue growth factor-2 sometimes hereinafter referred to as "CTGF-2". The invention also relates to inhibiting the action of such polypeptides.

The CTGF polypeptides are structurally and functionally related to a family of growth factors which include IGF (insulin-like growth factor), PDGF (platelet-derived growth factor), and FGF (fibroblast growth factor). This emerging family of secreted proteins are a group of cysteine-rich proteins. This group of growth factors are important for normal growth, differentiation, morphogenesis of the cartilaginous skeleton of an embryo and cell growth. Among some of the functions that have been discovered for these growth factors are wound healing, tissue repair, implant fixation and stimulating increased bone mass.

The extended superfamily of growth factors include TGF (transforming growth factor), bone morphogenic factors, and activins, among others.

The most well-known growth factor, TGF exerts a number of different effects on a variety of cells. For example, TGF-β can inhibit the diff rentiation of certain cells of mesodermal origin (Florini, J.R. et al., J. Biol. Chem., 261:1659-16513 (1986) induced the differentiation of others (Seyedine, S.M. et al., PNAS USA, 82:2267-2271 (1987) and potently inhibit proliferation of various types of epithelial cells, (Tucker, R.F., Science, 226:705-705 (1984)). This last activity has led to the speculation that one important physiological role for TGF-β is to maintain the repressed growth state of many types of cells. Accordingly, cells that lose the ability to respond to TGF-β are more likely to exhibit uncontrolled growth and become tumorigenic.

Accordingly, due to amino acid sequence homology the polypeptide of the present invention is a member of this extended family of growth factors which has many effects on a variety of different tissues.

In accordance with one aspect of the present invention, there is provided a novel mature polypeptide which is CTGF-2, as well as fragments, analogs and derivatives thereof. The polypeptide of the present invention is of human origin.

In accordance with another aspect of the present invention, there are provided polynucleotides (DNA or RNA) which encode such polypeptides.

In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptide by recombinant techniques.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptide, or polynucleotide encoding such polypeptide for therapeutic purposes, for example, enhancing the repair of connective and support tissue, promoting the attachment, fixation and stabilization of tissue implants and enhancing wound healing.

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In accordance with yet a further aspect of the present invention, there are provided antibodies against such polypeptides.

In accordance with yet another aspect of the present invention, there are provided antagonist/inhibitors to such polypeptides, which may be used to inhibit the action of such polypeptides, for example, in the treatment of CTGF dependent tumor growth.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 depicts the cDNA sequence and corresponding deduced amino acid sequence and corresponding letter abbreviation for amino of CTGF-2. The standard one-

Figure 2 is an amino acid comparison between CTGF-2 (top) and Cyr61 ((bottom) illustrating the amino sequence homology.

Figure 3 is an amino acid comparison between CTGF-2 (top) and mouse CTGF (bottom) illustrating the amino acid sequence homology.

Figure 4 depicts the results of a Northern Blot analysis of CTGF-2.

In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figure 1 or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. 75804.

The polynucleotide of this invention was discovered in a cDNA library derived from Human fetal lung. It is structurally related to the IGF and PDGF family. It contains an open reading frame encoding a protein of approximately 375

amino acid residues of which approximately the first 24 amino acids residues are the putative leader sequence such that the putative mature protein compris s 201 amino acids. The protein exhibits the highest degree f homology to Mouse CTGF with 49% identity and 67% similarity and to Cyr61 with 89% identity and 93% similarity. Cyr61 is a growth factor-inducible immediate early gene initially identified in serum-stimulated mouse fibroblasts. It encodes a member of an emerging family of cysteine-rich secreted proteins that includes a connective tissue growth factor (O'Brien and Lau, L.F., Cell Growth Differ., 3:645-54 (1992)).

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figure 1 or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature polypeptide as the DNA of Figure 1 or the deposited cDNA.

The polynucleotide which encodes for the mature polypeptide of Figure 1 or for the mature polypeptide encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding

sequence for the polyp ptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encod for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 1 or the polypeptide encoded by the cDNA of the deposited clone. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figure 1 or the same mature polypeptide encoded by the cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figure 1 or the polypeptide encoded by the cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexa-histidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian

host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitop derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The pr sent invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 50% and preferably 70% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides . As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNA of Figure 1 or the deposited cDNA.

The deposit(s) referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to a CTGF-2 polypeptide which has the deduced amino acid sequence of Figure 1 or which has the amino acid sequence encoded by the

deposited cDNA, as well as fragments, analogs and derivatives of such polyp ptide.

The terms "fragment," "derivative" and "analog" when referring to th polypeptide of Figure 1 or that encoded by the deposited cDNA, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The fragment, derivative or analog of the polypeptide of Figure 1 or that encoded by the deposited cDNA may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-

occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptid , s parated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the CTGF-2 genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies.

However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli. lac or trp, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as y ast; insect cells such as Drosophila and Sf9; animal cells such as CHO, COS or Bowes melanoma; plant cells, etc. The

selection of an appropriate host is deemed to be within the scope of th se skilled in the art from the teachings herein.

Mor particularly, the present invention also includes recombinant constructs comprising one or more of th sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pRG1, pD10, phagescript, psiX174, pBluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); pTrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are PKK232-8 and PCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast

cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphat transfection, DEAE-Dextran mediated transfection, or electroporation. (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a highly-expressed gene to direct

transcription of a downstream structural sequence. Such promoters can be derived from op rons ncoding glycolytic enzymes such as 3-phosphoglycerate kinas (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone"

sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitabl host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well know to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The CTGF-2 polypeptides can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography hydroxylapatite chromatography and

l ctin chromatography. Protein refolding steps can be used, as n c ssary, in completing configuration of the mature protein. Finally, high performanc liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

The CTGF-2 polypeptides of the present invention may be used to enhance the repair of connective and support tissue. For example CTGF-2 may be used to treat skin disorders such as injuries, acne, aging, UV damage or burns. CTGF-2 may also be used to improve the cosmetic appearance of the skin, for example, by treating wrinkled skin.

CTGF-2 may also be used to promote the attachment, fixation and stabilization of tissue implants, for example, a prosthesis and other implants inserted during reconstructive surgery. The CTGF-2 polypeptide of the present invention may be used in the healing of external wounds, by promoting growth of epithelial and connective tissues and the synthesis of total protein and collagen. CTGF-2 may be applied in the area of injured or depleted bones, with regeneration occurring by promoting the growth of connective tissue, bone and cementum and by stimulating protein and collagen synthesis which is especially useful for periodontal disease.

The polypeptide of the present invention is also useful for identifying other molecules which have similar biological activity. For example, the coding region of the CTGF-2 gene

is isolated by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA to determine which members of the library the probe hybridizes to.

The polypeptides may also be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus which may be used to engineer cells in vivo after combination with a suitable delivery vehicle.

The polypeptides of the present invention may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the polypeptide, and a pharmaceutically

acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the polypeptides of the present invention may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. CTGF-2 is administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, CTGF-2 will be administered in an amount of at least about 10 μg/kg body weight and in most cases CTGF-2 will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10 μg/kg to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step

in correlating those sequences with genes associated with dis ase.

Briefly, sequences can be mapp d to chromosomes by pr paring PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the cDNA is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clones to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 500 or 600 bases; however, clones larger than 2,000 bp have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. FISH requires use of the clones from which the EST was derived, and the longer the better. For example, 2,000 bp is good, 4,000 is better, and more than 4,000 is probably not necessary to get good results a reasonable percentage of the time. For a review of this technique, see Verma et al., Human Chromosomes: a Manual of Basic Techniques, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromos me can be correlated with genetic map data. Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then

bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention.

This invention provides a method for identification of the receptor for the CTGF-2 polypeptide. The gene encoding the receptor can be identified by expression cloning. Briefly, polyadenylated RNA is prepared from a cell responsive to CTGF-2, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to CTGF-2. Transfected cells which are grown on glass slides are exposed to labeled CTGF-2. The CTGF-2 can be labeled by a variety of means including iodidation or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to autoradiographic analysis. Positive pools are identified and sub-pools are prepared and retransfected using an iterative sub-pooling and rescreening process, eventually yielding a single clone that encodes the putative receptor. As an alternative approach for receptor identification, labeled ligand can be

photoaffinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE and exposed to x-ray film. The labeled complex containing the CTGF-2-receptor can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of generate oligonucleotide probes to screen a cDNA library to identify the gene encoding the putative receptor.

This invention also provides a method of screening drugs to identify those which enhance or block interaction of CTGF-2 with its receptor. An agonist is a compound which increases the natural biological functions of CTGF-2 and an antagonist inhibits these functions. As an example, a mammalian cell or membrane preparation expressing the CTGF-2 receptor would be incubated with labeled CTGF-2 in the presence of drug. The ability of the drug to enhance or block this interaction could then be measured. Alternatively, the response of a known second messenger system following interaction of CTGF-2 and its receptor would be measured compared in the presence or absence of the drug. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis.

The present invention is also directed to antagonist/inhibitor molecules of the polypeptides of the present invention, and their use in reducing or eliminating the function of CTGF-2.

An example of an antagonist is an antibody or in some cases, an oligonucleotide, which binds to the CTGF-2 polypeptide. Alternatively, antagonists include closely related proteins that have lost biological function and thereby prevent the action of CTGF-2 since receptor sites are occupied.

Antisense technology may be employed to decrease the level of *in vivo* circulation of CTGF-2. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix - see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991)), thereby preventing transcription and the production of CTGF-2. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into CTGF-2 (antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of CTGF-2.

An example of an inhibitor is a small molecule which binds to the CTGF-2 receptors such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

The antagonist/inhibitors may be employed to prevent scar formation due to excess proliferation of connective tissues and to prevent CTGF-2 dependent tumor growth. The antagonist/inhibitors may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinabove described.

The present invention also relates to an assay for identifying potential antagonist/inhibitors specific to CTGF-

2. An example of such an assay combines CTGF-2 and a potential antagonist/inhibitor with membrane-bound CTGF-2 receptors or recombinant CTGF-2 under appropriate conditions for a competitive inhibition assay. CTGF-2 can be labeled, such as by radio activity, such that the number of CTGF-2 molecules bound to the receptor can determine the effectiveness of the potential antagonist/inhibitor.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 μg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 μl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for

particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. *et al.*, Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units to T4 DNA ligase ("ligase") per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, F. and Van der Eb, A., Virology, 52:456-457 (1973).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: LI, ET AL.

(ii) TITLE OF INVENTION: Connective Tissue Growth Factor-2

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN, CECCHI, STEWART & OLSTEIN
(B) STREET: 6 BECKER FARM ROAD
(C) CITY: ROSELAND
(D) STATE: NEW JERSEY
(E) COUNTRY: USA
(F) ZIP: 07068

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 INCH DISKETTE
(B) COMPUTER: IBM PS/2
(C) OPERATING SYSTEM: MS-DOS
(D) SOFTWARE: WORD PERFECT 5.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: Concurrently
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: FERRARO, GREGORY D.
(B) REGISTRATION NUMBER: 36,134
(C) REFERENCE/DOCKET NUMBER: 325800-129

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 201-994-1700
(B) TELEFAX: 201-994-1744

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 1128 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATGAGCTCCC GAATCGTCAG GGAGCTCGCC TTAGTCGTCA CCCTTCTCCA CTTGACCAGG      60
GTGGGGCTCT CCACCTGCCC CGCTGACTGC CACTGCCCCC TGGAGGCGCC CAAGTGCGCG     120
CCGGGAGTCG GGCTGGTCCG GGACGGCTGC GGCTGTTGTA AGGTCTGCGC CAAGCAGCTC     180
AACGAGGACT GCAGAAAAAC GCAGCCCTGC GACCACACCA AGGGGCTGGA ATGCAACTTC     240
GGCGCCAGCT CCACCGCTCT GAAGGGGATC TGCAGAGCTC AGTCAGAGGG CAGACCCTGT     300
GAATATAACT CCAGAATCTA CCAAAACGGG GAAAGTTTCC AGCCCAACTG TAAACATCAG     360
TGCACATGTA TTGGATGGCG CCGGGGGGCT TGCATTCCTC TGTGTCCCCA AGAACTATCT     420
CCCCCCAACT TGGGCTGTCC CAACCCTCGG CTGGTCAAAG TTACCGGGCA GTGCTGCGAG     480
GAGTGGGTCT GTGACGAGGA TAGTATCAAG GACCCCATGG AGGACCAGGA CGGCCTCCTT     540
GGCAAGGGGC TGGGATTCGA TGCCTCCGAG GTGGAGTTGA CGAGAAACAA TGAATTGATT     600
GCAGTTGGAA AAGGCAGCTC ACTGAAGCGG CTCCCTGTTT TTGGAATGGA GCCTCGCATC     660
CTATACAACC CTTTACAAGG CCAGAAATGT ATTGTTCAAA CAACTTCATG GTCCCAGTGC     720
TCAAAGACCT GTGGAACTGG TATCTCCACA CGAGTTACCA ATGACAACCC TGAGTGCCGC     780
CTTGTGAAAG AAACCCGGAT TTGTGAGGTG CGGCCTTGTG GACAGCCAGT GTACAGCAGC     840
CTGAAAAAGG GCAAGAAATG CAGCAAGACC AAGAAATCCC CCGAACCAGT CAGGTTTACT     900
TACGCTGGAT GTTTGAGTGT GAAGAAATAC CGGCCCAAGT ACTGCGGTTC CTGCGTGGAC     960
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```
GGCCGATGCT GCACGCCCCA GCTGACCAGG ACTGTGAAGA TGCGGTTCCC CTGCGAAGAT      1020
GGGGAGACAT TTTCCAAGAA CGTCATGATG ATCCAGTCCT CCAAATGCAA CTACAACTGC      1080
CCGCATGCCA ARGAAGCAGC GTTTCCCTTC TACAGGCTGT TCCAATGA                   1128
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 375 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS:

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ser Ser Arg Ile Val Arg Glu Leu Ala Leu Val Val Thr Leu
                -20                 -15                 -10
Leu His Leu Thr Arg Val Gly Leu Ser Thr Cys Pro Ala Asp Cys
                 -5                  1                   5
His Cys Pro Leu Glu Ala Pro Lys Cys Ala Pro Gly Val Gly Leu
             10                  15                  20
Val Arg Asp Gly Cys Gly Cys Cys Lys Val Cys Ala Lys Gln Leu
             25                  30                  35
Asn Glu Asp Cys Arg Lys Thr Gln Pro Cys Asp His Thr Lys Gly
             40                  45                  50
Leu Glu Cys Asn Phe Gly Ala Ser Ser Thr Ala Leu Lys Gly Ile
             55                  60                  65
Cys Arg Ala Gln Ser Glu Gly Arg Pro Cys Glu Tyr Asn Ser Arg
             70                  75                  80
Ile Tyr Gln Asn Gly Glu Ser Phe Gln Pro Asn Cys Lys His Gln
             85                  90                  95
Cys Thr Cys Ile Gly Thp Arg Arg Gly Ala Cys Ile Pro Leu Cys
            100                 105                 110
Pro Gln Glu Leu Ser Leu Pro Asn Leu Gly Cys Pro Asn Pro Arg
            115                 120                 125
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```
Leu Val Lys Val Thr Gly Gln Cys Cys Glu Glu Trp Val Cys Asp
            130                 135                 140
Glu Asp Ser Ile Lys Asp Pro Met Glu Asp Gln Asp Gly Leu Leu
            145                 150                 155
Gly Lys Gly Leu Gly Phe Asp Ala Ser Glu Val Glu Leu Thr Arg
            160                 165                 170
Asn Asn Glu Leu Ile Ala Val Gly Lys Gly Ser Ser Leu Lys Arg
            175                 180                 185
Leu Pro Val Phe Gly Met Glu Pro Arg Ile Leu Tyr Asn Pro Leu
            190                 195                 200
Gln Gly Gln Lys Cys Ile Val Gln Thr Thr Ser Trp Ser Gln Cys
            205                 210                 215
Ser Lys Thr Cys Gly Thr Gly Ile Ser Thr Arg Val Thr Asn Asp
            220                 225                 230
Asn Pro Glu Cys Arg Leu Val Lys Glu Thr Arg Ile Cys Gly Val
            235                 240                 245
Arg Pro Cys Gly Gln Pro Val Tyr Ser Ser Leu Lys Lys Gly Lys
            250                 255                 260
Lys Cys Ser Lys Thr Lys Lys Ser Pro Glu Pro Val Arg Phe Thr
            265                 270                 275
Tyr Ala Gly Cys Leu Ser Val Lys Lys Tyr Arg Pro Lys Tyr Cys
            280                 285                 290
Gly Ser Cys Val Asp Gly Arg Cys Cys Thr Pro Gln Leu Thr Arg
            295                 300                 305
Thr Val Lys Met Arg Phe Pro Cys Glu Asp Gly Glu Thr Phe Ser
            310                 315                 320
Lys Asn Val Met Met Ile Gln Ser Ser Lys Cys Asn Tyr Asn Cys
            325                 330                 335
Pro His Ala Asn Glu Ala Ala Phe Pro Phe Tyr Arg Leu Phe Gln
            340                 345                 350
```

Example 1

Cloning and expression of CTGF-2 in a baculovirus expression system

The DNA sequence encoding the full length CTGF-2 protein, ATCC # 75804, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:
The 5' primer has the sequence CGCG**GGATCC**TGCGCGACACAATGAGCT and contains a BamHI restriction enzyme site (in bold) followed by 18 nucleotides resembling an efficient signal for the initiation of translation in eukaryotic cells (J. Mol. Biol. 1987, 196, 947-950, Kozak, M.). The initiation codon for translation "ATG" is underlined.

The 3' primer has the sequence CGCG**GGTACC**AGGTAGCATTTAGTCCCTAA and contains the cleavage site for the restriction endonuclease Asp781 and 20 nucleotides complementary to the 3' non-translated sequence of the CTGF-2 gene. The amplified sequences were isolated from a 1% agarose gel using a commercially available kit ("Geneclean", BIO 101 Inc., La Jolla, Ca.). The fragment was then digested with the endonucleases BamHI and Asp781 and then purified by isolation on a 1% agarose gel. This fragment is designated F2.

The vector pRG1 (modification of pVL941 vector, discussed below) is used for the expression of the CTGF-2 protein using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases BamHI and Asp781. The polyadenylation site of the simian virus (SV)40 is used for efficient polyadenylation. For an easy selection of recombinant viruses the beta-galactosidase gene from E.coli is inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by viral sequences for the cell-mediated homologous recombination of cotransfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pRG1

such as pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., Virology, 170:31-39).

The plasmid was digested with the restriction enzymes BamHI and Asp781 and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The DNA was then isolated from a 1% agarose gel. This vector DNA is designated V2.

Fragment F2 and the dephosphorylated plasmid V2 were ligated with T4 DNA ligase. E.coli HB101 cells were then transformed and bacteria identified that contained the plasmid (pBacCTGF-2) with the CTGF-2 gene using the enzymes BamHI and Asp781. The sequence of the cloned fragment was confirmed by DNA sequencing.

5 µg of the plasmid pBacCTGF-2 were cotransfected with 1.0 µg of a commercially available linearized baculovirus ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofection method (Felgner et al. Proc. Natl. Acad. Sci. USA, 84:7413-7417 (1987)).

1µg of BaculoGold™ virus DNA and 5 µg of the plasmid pBacCTGF-2 were mixed in a sterile well of a microtiter plate containing 50 µl of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 µl Lipofectin plus 90 µl Grace's medium were added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture was added dropwise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1ml Grace' medium without serum. The plate was rocked back and forth to mix the newly added solution. The plate was then incubated for 5 hours at 27°C. After 5 hours the transfection solution was removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum was added. The plate was put back into an incubator and cultivation continued at 27°C for four days.

After four days the supernatant was collected and a plaque assay performed similar as described by Summers and Smith (supra). As a modification an agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) was used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be

found in the us r's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution of the viruses was added to the cells, blue stained plaques were picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses was then resuspended in an Eppendorf tube containing 200 µl of Grace's medium. The agar was removed by a brief centrifugation and the supernatant containing the recombinant baculoviruses was used to insect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes were harvested and then stored at 4°C.

Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells were infected with the recombinant baculovirus V-CTGF-2 at a multiplicity of infection (MOI) of 2. Six hours later the medium was removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Gaithersburg). 42 hours later 5 µCi of ^{35}S-methionine and 5 µCi ^{35}S cysteine (Amersham) were added. The cells were further incubated for 16 hours before they were harvested by centrifugation and the labelled proteins visualized by SDS-PAGE and autoradiography.

Example 2

Expression of Recombinant CTGF-2 in COS cells

The expr ssion of plasmid, CTGF-2 HA is deriv d from a vector pcDNAI/Amp (Invitrogen) containing: 1) SV40 origin of r plication, 2) ampicillin resistance gene, 3) E.coli replication origin, 4) CMV promoter followed by a polylinker region, a SV40 intron and polyadenylation site. A DNA fragment encoding the entire CTGF-2 precursor and a HA tag fused in frame to its 3' end was cloned into the polylinker region of the vector, therefore, the recombinant protein expression is directed under the CMV promoter. The HA tag correspond to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, H. Niman, R. Heighten, A Cherenson, M. Connolly, and R. Lerner, 1984, Cell 37, 767). The infusion of HA tag to our target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follows:

The DNA sequence encoding for CTGF-2, ATCC # 75804, was constructed by PCR on the full-length clone using two primers: the 5' primer 5' AAAGGATCCACAATGAGCTCCCGAATC 3' contains a Bam HI site followed by 18 nucleotides of CTGF-2 coding sequence starting from the -3 position relative to the initiation codon; the 3' sequence 5' CGCTCTAGATTAAGCGTAGTCTGGGACGTCGTATGGGTATTGGAACAGCCTGTAGAAG 5' contains complementary sequences to an Xba I site, translation stop codon, HA tag and the last 19 nucleotides of the CTGF-2 coding sequence (not including the stop codon). Therefore, the PCR product contains a Bam HI site, CTGF-2 coding sequence followed by an HA tag fused in frame, a translation termination stop codon next to the HA tag, and an Xba I site. The PCR amplified DNA fragment and the vector, pcDNAI/Amp, were digested with Bam HI and Xba I restriction enzymes and ligated. The ligation mixture was transformed into E. coli strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037) the transformed culture was plated on ampicillin media plates and resistant colonies were selected. Plasmid DNA was isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the

recombinant CTGF-2, COS cells were transfected with the expression vector by DEAE-DEXTRAN method. (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). Th expression of the CTGF-2 HA protein was detected by radiolabelling and immunoprecipitation method. (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells were labelled for 8 hours with ^{35}S-cysteine two days post transfection. Culture media were then collected and cells were lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5). (Wilson, I. et al., Id. 37:767 (1984)). Both cell lysate and culture media were precipitated with a HA specific monoclonal antibody. Proteins precipitated were analyzed on 15% SDS-PAGE gels.

Example 3

Expression pattern of CTGF-2 in human tissue

Northern blot analysis was carried out to examine the levels of expression of CTGF-2 in human tissues. Total cellular RNA samples were isolated with RNAzol™ B system (Biotecx Laboratories, Inc. 6023 South Loop East, Houston, TX 77033). About 10μg of total RNA isolated from each human tissue specified was separated on 1% agarose gel and blotted onto a nylon filter. (Sambrook, Fritsch, and Maniatis, Molecular Cloning, Cold Spring Harbor Press, (1989)). The labeling reaction was done according to the Stratagene Prime-It kit with 50ng DNA fragment. The labeled DNA was purified with a Select-G-50 column. (5 Prime - 3 Prime, Inc. 5603 Arapahoe Road, Boulder, CO 80303). The filter was then hybridized with radioactive labeled full length CTGF-2 gene at 1,000,000 cpm/ml in 0.5 M $NaPO_4$, pH 7.4 and 7% SDS overnight at 65°C. After wash twice at room temperature and twice at 60°C with 0.5 x SSC, 0.1% SDS, the filter was then exposed at -70°C overnight with an intensifying screen. The message RNA for CTGF-2 is abundant in the heart. (Figure 3).

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within

the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

WHAT IS CLAIMED IS:

1. An isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide encoding a CTGF-2 polypeptide having the deduced amino acid sequence of Figure 1 or a fragment, analog or derivative of said polypeptide;

(b) a polynucleotide encoding a CTGF-2 polypeptide having the amino acid sequence encoded by cDNA contained in ATCC Deposit No. 75804 or a fragment, analog or derivative of said polypeptide.

2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.

3. The polynucleotide of Claim 1 wherein the polynucleotide is RNA.

4. The polynucleotide of Claim 1 wherein the polynucleotide is genomic DNA.

5. The polynucleotide of Claim 2 wherein said polynucleotide encodes CTGF-2 having the deduced amino acid sequence of Figure 1.

6. The polynucleotide of Claim 2 wherein said polynucleotide encodes a CTGF-2 polypeptide encoded by the cDNA of ATCC Deposit No. 75804.

7. The polynucleotide of Claim 1 having the coding sequence of CTGF-2 as shown in Figure 1.

8. The polynucleotide of Claim 2 having the coding sequence of CTGF-2 deposited as ATCC Deposit No. 75804.

9. A vector containing the DNA of Claim 2.

10. A host cell genetically engineered with the vector of Claim 9.

11. A process for producing a polypeptide comprising: expressing from the host cell of Claim 10 the polypeptide encoded by said DNA.

12. A process for producing cells capable of expressing a polypeptide comprising genetically engineering cells with the vector of Claim 9.

13. An isolated DNA hybridizable to the DNA of Claim 2 and encoding a polypeptide having CTGF-2 activity.

14. A polypeptide s lected from the group consisting of (i) a CTGF-2 polypeptide having the deduced amino acid sequence of Figure 1 and fragments, analogs and derivatives thereof and (ii) a CTGF-2 polypeptide encoded by the cDNA of ATCC Deposit No. 75804 and fragments, analogs and derivatives of said polypeptide.

15. The polypeptide of Claim 14 wherein the polypeptide is CTGF-2 having the deduced amino acid sequence of Figure 1.

16. An antibody against the polypeptide of claim 14.

17. An antagonist/inhibitor against the polypeptide of claim 14.

18. A method for the treatment of a patient having need of CTGF-2 comprising: administering to the patient a therapeutically effective amount of the polypeptide of claim 14.

19. A method for the treatment of a patient having need to inhibit CTGF-2 comprising: administering to the patient a therapeutically effective amount of the antagonist/inhibitor of Claim 17.

20. The method of Claim 18 wherein said therapeutically effective amount of the polypeptide is administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.

FIG. 1A

```
ATGAGCTCCCGAATCGTCAGGGAGCTCGCCTTAGTCGTCACCCTTCTCCACTTGACCAGG
M  S  S  R  I  V  R  E  L  A  L  V  V  T  L  L  L  H  L  T  R

GTGGGGCTCTCCACCTGCCCCGCTGACTGCCACTGCCCCCCTGGAGGCGCCCAAGTGCGCG
V  G  L  S  T  C  P  A  D  C  H  C  P  L  E  A  P  K  C  A

CCGGGAGTCGGGCTGGTCCGGGACGGCTGCGGCTGTTGTAAGGTCTGCGCCAAGCAGCTC
P  G  V  G  L  V  R  D  G  C  G  C  C  K  V  C  A  K  Q  L

AACGAGGACTGCAGAAAACGCAGCCCTGCGACCACACCAAGGGGCTGGAATGCAACTTC
N  E  D  C  R  K  T  Q  P  C  D  H  T  K  G  L  E  C  N  F

GGCGCCAGCTCCACCGCTCTGAAGGGGATCTGCAGAGCTCAGTCAGAGGGCAGACCCTGT
G  A  S  S  T  A  L  K  G  I  C  R  A  Q  S  E  G  R  P  C

GAATATAACTCCAGAATCTACCAAAACGGGGAAAGTTTCCAGCCCAACTGTAAACATCAG
E  Y  N  S  R  I  Y  Q  N  G  E  S  F  Q  P  N  C  K  H  Q

TGCACATGTATTGGATGGCGCCGGGGGGCTTGCATTCCTCTGTGTCCCCAAGAACTATCT
C  T  C  I  G  W  R  R  G  A  C  I  P  L  C  P  Q  E  L  S

CTCCCCAACTTGGGCTGTCCCAACCCTCGGCTGGTCAAAGTTACCGGGCAGTGCTGCGAG
L  P  N  L  G  C  P  N  P  R  L  V  K  V  T  G  Q  C  C  E
```

MATCH WITH FIG. 1B

MATCH WITH FIG. IA

FIG. IB

```
GAGTGGGTCTGTGACGAGGATAGTATCAAGGACCCCATGGAGGACCAGGACGGCCTCCTT
E  W  V  C  D  E  D  S  I  K  D  P  M  E  D  Q  D  G  L  L

GGCAAGGGGCTGGGATTCGATGCCTCCGAGGTGGAGTTGACGAGAAACAATGAATTGATT
G  K  G  L  G  F  D  A  S  E  V  E  L  T  R  N  N  E  L  I

GCAGTTGGAAAAGGCAGCTCACTGAAGCGGCTCCCTGTTTTTGGAATGGAGCCTCGCATC
A  V  G  K  G  S  S  L  K  R  L  P  V  F  G  M  E  P  R  I

CTATACAACCCTTTACAAGGCCAGAAATGTATTGTTCAAACAACTTCATGGTCCCAGTGC
L  Y  N  P  L  Q  G  Q  K  C  I  V  Q  T  T  S  W  S  Q  C

TCAAAGACCTGTGGAACTGGTATCTCCACACGAGTTACCAATGACAACCCTGAGTGCCGC
S  K  T  C  G  T  G  I  S  T  R  V  T  N  D  N  P  E  C  R

CTTGTGAAAGAAACCCGGATTTGTGAGGTGCGGCCTTGTGGACAGCCAGTGTACAGCAGC
L  V  K  E  T  R  I  C  E  V  R  P  C  G  Q  P  V  Y  S  S

CTGAAAAAGGGCAAGAAATGCAGCAAGACCAAGAAATCCCCCGAACCAGTCAGGTTTACT
L  K  K  G  K  K  C  S  K  T  K  K  S  P  E  P  V  R  F  T
```

MATCH WITH FIG. IC

MATCH WITH FIG. 1B

FIG. 1C

```
TACGCTGGATGTTTGAGTGTGAAGAAATACCGGCCCAAGTACTGCGGTTCCTGCGTGGAC
Y  A  G  C  L  S  V  K  K  Y  R  P  K  Y  C  G  S  C  V  D

GGCCGATGCTGCACGCCCCAGCTGACCAGGACTGTGAAGATGCGGTTCCCCTGCGAAGAT
G  R  C  C  T  P  Q  L  T  R  T  V  K  M  R  F  P  C  E  D

GGGGAGACATTTTCCAAGAACGTCATGATGATCCAGTCCTCCAAATGCAACTACAACTGC
G  E  T  F  S  K  N  V  M  M  I  Q  S  S  K  C  N  Y  N  C

CCGCATGCCAATGAAGCAGCGTTTCCCTTCTACAGGCTGTTCCAATGA
P  H  A  N  E  A  A  F  P  F  Y  R  L  F  Q  *
```

FIG. 2A

```
  1 MSSRIVRELALVVTLLHLTRVGLSTCPADCHCPLEAPKCAPGVGLVRDGC 50
    |||...|.||:.|||||||::||||||.||||||||||||||||||||||
  1 MSSSTFRTLAVAVTLLHLTRLALSTCPAACHCPLEAPKCAPGVGLVRDGC 50

 51 GCCKVCAKQLNEDCRKTQPCDHTKGLECNFGASSTALKGICRAQSEGRPC 100
    ||||||||||||||.||||||||||||||||||||||||||||||||||||
 51 GCCKVCAKQLNEDCSKTQPCDHTKGLECNFGASSTALKGICRAQSEGRPC 100

101 EYNSRIYQNGESFQPNCKHQCTCIGWRRGACIPLCPQELSLPNLGCPNPR 150
```

MATCH WITH FIG. 2B

FIG. 2B

MATCH WITH FIG. 2A

```
101 EYNSRIYQNGESFQPNCKHQCTCID.GAVGCIPLCPQELSLPNLGCPNPR 149

151 LVKVTGQCCEEWVCDEDSIKDPMEDQDGLLGKGLGFDASEVELTRNNELI 200
150 LVKVSGQCCEEWVCDEDSIKDSLDDQDDL....LGLDASEVELTRNNELI 195

201 AVGKGSSLKRLPVFGMEPRILYNPL..QGQKCIVQTTSWSQCSKTCGTGI 248
196 AIGKGSSLKRLPVFGTEPRVLFNPLHAHGQKCIVQTTSWSQCSKSCGTGI 245

249 STRVTNDNPECRLVKETRICEVRPCGQPVYSSLKKGKKCSKTKKSPEPVR 298
246 STRVTNDNPECRLVKETRICEVRPCGQPVYSSLKKGKKCSKTKKSPEPVR 295

299 FTYAGCLSVKKYRPKYCGSCVDGRCCTPQLTRTVKMRFPCEDGETFSKNV 348
296 FTYAGCSSVKKYRPKYCGSCVDGRCCTPLQTRTVKMRFRCEDGEMFSKNV 345

349 MMIQSSKCNYNCPHANEAAFPFYRLFQ 375
346 MMIQSCKCNYNCPHPNEASFRLYSLFN 372
```

FIG. 3

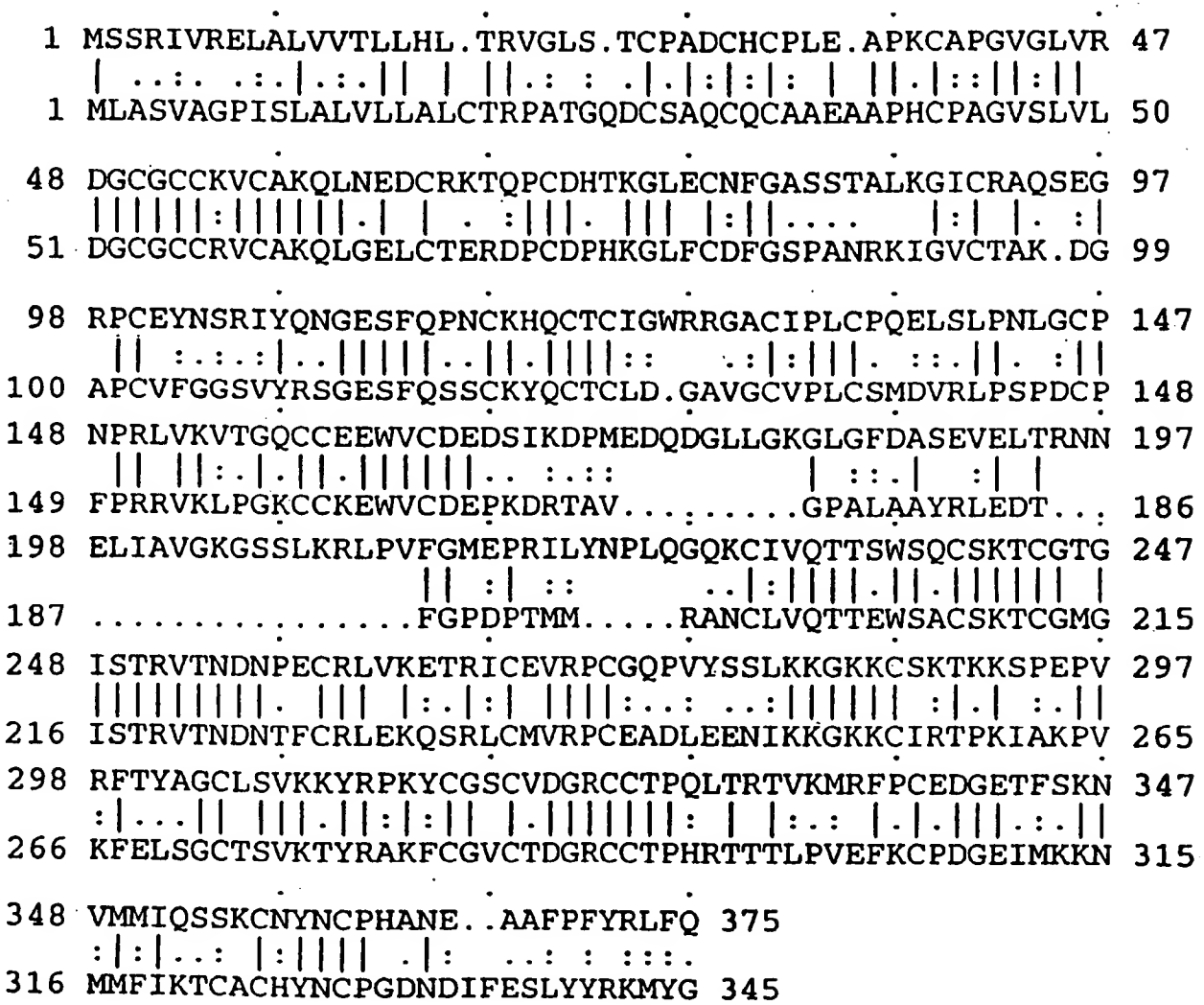

FIG. 4

1 = overy 2=testis 3 = gall blader 4 = kidney 5 = liver 6 = lung

7 = spleen 8 = prostate 9 = hippocampus 10 = heart 11 = pancrease

12 = placenta 13 = thymus

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07736

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/00, 15/18; C07K 14/00; A61K 38/27

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.3, 240.2, 252.3, 252.33, 320.1; 514/2, 12; 530/350, 399; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and DIALOG (files 5, 155, 351, 357, 358) search terms: CTGF, CTGF-2, connective tissue growth factor, osteogenic, bone

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF CELL BIOLOGY, Volume 114, Number 6, issued September 1991, Bradham et al, "Connective Tissue Growth Factor: a Cysteine-rich Mitogen Secreted by Human Vascular Endothelial Cells Is Related to the SRC-induced Immediate Early Gene Product CEF-10", pages 1285-1294, see entire document.	1-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 OCTOBER 1994	28 NOV 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MARIANNE PORTA ALLEN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07736

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-15

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07736

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 172.3, 240.2, 252.3, 252.33, 320.1; 514/2, 12; 530/350, 399; 536/23.5

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I. Claims 1-15, drawn to a polynucleotide sequence, vector, host cell, process of producing the host cell, process of producing the protein, and protein, classified in at least Class 536, subclass 23.5, for example.

Group II. Claim 16, drawn to an antibody, classified in at least Class 530, subclass 387.1, for example.

Group III. Claim 17, drawn to an antagonist, classified in at least Class 530, subclass 350, for example.

Group IV. Claims 18-20, drawn to methods of treatment, classified in at least Class 514, subclass 44, for example.

The claims of the four groups are directed to different inventions which are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept. Note that PCT Rule 13 does not provide for multiple products or methods within a single application.